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FULL RESEARCH PAPER

Development of real-time PCR systems based on SYBR® Green I and TaqMan® technologies for specific quantitative detection of *Phoma tracheiphila* in infected *Citrus*

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Abstract Real-time PCR assays based on SYBR® Green I and TaqMan® technologies were developed for *in planta* detection and quantification of *Phoma tracheiphila*, the mitosporic fungus causing 'mal secco' disease on citrus. Primers and a hybridization probe were designed on the basis of the internal transcribed spacer (ITS) region of the nuclear rRNA genes. The real-time PCR assays were compared with a classic isolation method in two separate experiments carried out on 6 and 24 month-old sour orange seedlings, artificially inoculated with a conidial suspension of the pathogen. Both technologies made it possible to follow the progression of infection by *P. tracheiphila*, enabling detection and quantification of the target fungus prior to the development of symptoms. The detection limit was 10 copies of the cloned target sequence and 15 pg of genomic DNA extracted from fungal spores. The values of the cycle threshold (Ct) were linearly correlated with the concentration of the target DNA, indicating that the method is suitable as a qualitative and quantitative assay. The presence of non-target fungal DNA had no effect on the specificity of the assay, but resulted in a 10-fold reduction of sensitivity. Total inhibition of the reaction occurred when conidia of the target pathogen were mixed with an organic soil substrate before extracting DNA using the standard protocol, while an alternative purification kit resulted in a significant decrease in sensitivity. Compared to classic methods, real-time PCR proved faster and easier to perform and showed a higher sensitivity. These results suggest that real-time PCR, based on both chemistries, has a great potential for early diagnosis of 'mal secco' disease and for quantitative estimation of fungal growth within host tissue.

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Introduction

Phoma tracheiphila is a mitosporic fungus causing a destructive vascular disease of *Citrus* known as 'mal secco' (Baldacci and Garofalo 1948; Nachmias et al. 1979; Salerno and Perrotta 1966; Solel and Salerno 2000). 'Mal secco' disease affects species of the following genera: *Citrus, Fortunella, Poncirus* and *Severinia*, but it is particularly severe on lemon (*Citrus limon*). Up to 100% of trees in an orchard of a susceptible lemon cultivar can be affected. Destructive outbreaks of this disease may occur after frost spells and hail storms (CABI/EPPO, 1997). 'Mal secco' disease reduces lemon production and limits the use of susceptible species and cultivars in areas where the disease pressure is high. In some Mediterranean regions a high incidence of 'mal secco' disease has made lemon culture economically marginal and at present no effective means are available to control it (OEPP/EPPO 2005). Lemon cultivars with some degree of resistance to 'mal secco' disease produce fruits of lower commercial quality. This serious disease of lemon is widespread throughout the Mediterranean region, including the Black Sea area, with the exception of a few countries (Punithalingam and Holliday 1973; Perrotta and Graniti 1988). The disease does not occur in the citrus-growing countries of the Americas and Oceania even though there is no obvious climatic or cultural factor limiting the potential establishment of 'mal secco' disease in uninfected areas. The European and Mediterranean Plant Protection Organization (OEPP/EPPO) has included *P. tracheiphila* in the A2 quarantine list of pests and diseases. Furthermore, *P. tracheiphila* is of quarantine concern to most other regional plant protection organizations, such as APPC, CPPC, COSAVE, APSC, NAPPO (CABI/EPPO 1997). Preventive measures based on early diagnosis are the most effective ways to limit the introduction and further spread of the pathogen.

P. tracheiphila is often present in latent infections, which can, in some cases, later develop into damaging symptomatic infections in the plant. As a result, an apparently healthy host may suddenly show all the symptoms of the disease and collapse. Until now the identification of *P. tracheiphila* has relied on conventional methods described in the OEPP/EPPO standards (http://www.eppo.org/STANDARDS/standards.htm). According to the OEPP/EPPO (2005) standard, diagnosis of 'mal secco' disease is considered positive when the fungus is isolated on agar media and identified on the basis of cultural and morphological characters, or on both the morphology and using a molecular method. The disadvantage of this approach is that detection of the pathogen is only possible at a late stage of the infection, when it is already too late for any countermeasure to be taken, and the epidemic spread of the disease can no longer be controlled. The only molecular methods considered by the EPPO standard for *P. tracheiphila* detection consist in a dot blot assay and a polymerase chain reaction (PCR) test developed by Rollo and co-workers (Rollo et al. 1987, 1990). However, the latter method proved unreliable for routine diagnosis, giving rise to a series of non-specific amplimers when tested with template DNA from representatives of several fungal species (Balmas et al. 2005). While the EPPO standard was in press, a PCR-based specific assay coupled to electrophoretic separation of amplicons made it possible to detect *P. tracheiphila* in naturally infected *Citrus* wood tissues collected from both symptomatic and symptomless plants (Balmas et al. 2005). A pair of *P. tracheiphila*-specific primers (PtFOR2 and PtREV2) was designed based on the consensus sequence obtained from the alignment of the internal transcribed spacer (ITS) region of the nuclear rRNA genes of 17 *P. tracheiphila* isolates (GenBank accession numbers: AY531665 to AY531682 and AY531689) and of single representatives of six additional *Phoma* species [AY531683, AY531684, AY531685, AY531686, AY531687, AY531688 (*P. glomerata*, *P. exigua*, *P. betae*, *P. cava*, *P. fimeti*, and *P. lingam* isolates, respectively)] (Balmas et al. 2005). A PCR assay using primers PtFOR2 and PtREV2 enabled detection of the fungus in symptomless twigs (Balmas et al. 2005). Moreover, this method was found to be effective for the diagnosis of 'mal secco' infections in the hardwood of trees affected by 'mal nero,' a chronic *facies* of the disease (Balmas et al. unpublished results). The ability to detect infections in lignified citrus samples corroborates the robustness of this molecular method.

In previous studies, a relationship between symptom expression in 'mal secco' infected plants and the capability of *P. tracheiphila* to invade the vascular system

has been observed. In artificially inoculated plants, a close direct correlation was found between symptom severity and the rate of xylem colonisation by the fungus, as determined by the traditional isolation method on an agar-medium (Magnano di San Lio et al. 1992; Cacciola et al. 1996). Similarly, in other hadromycoses such as *Fusarium* and *Verticillium* wilt diseases of olive, tomato and cotton, the extent of vascular colonisation by the pathogen was correlated with the level of genotypic susceptibility of the host cultivar to the disease (Harrison and Beckman 1982; Gao et al. 1995; Mercado-Blanco et al. 2003). Therefore, a quantitative and rapid detection method to assess the rate of xylem colonisation could be a useful tool for evaluating the susceptibility to the disease during citrus selection and breeding programmes for 'mal secco' disease resistance.

In order to study the factors affecting the progression from latent to symptomatic disease, it would be essential to use of a sensitive and reliable method which would make it possible to monitor and quantify the presence of the fungus in plant tissues before symptoms appear. Recent advances in DNA-based techniques such as real-time PCR technologies (Livak et al. 1995) provide new tools for testing pathogens by detecting and accurately quantifying their DNA/RNA. These methods proved particularly useful to locate latent infections or to measure disease development before symptoms become visible (see for review: Lopez et al. 2003; Schaad and Frederick 2002; Ward et al. 2004).

Here we describe a fast and reliable method for specific identification and absolute quantification of *P. tracheiphila in planta* by a real-time PCR assay using two different technologies: the SYBR® Green I detection dye and a TaqMan® hybridisation probe. We tested the assays on plant material from sour orange artificially infected with *P. tracheiphila*, by comparing the results obtained with the real-time approach with those achieved by conventional isolation methods.

Materials and methods

Fungal isolates and storage conditions

The highly virulent, chromogenic isolate of *P. tracheiphila* FC40 (= ITEM 2338; Fogliano et al. 1998) obtained from diseased lemon (*Citrus limon*) was used in the plant inoculation experiments. Genomic DNA from different *Phoma* species, including *P. tracheiphila*, *P. betae*, *P. cava*, *P. exigua*, *P. fimeti*, *P. glomerata*, *P. lingam*, and *P. medicaginis*, and from other fungi commonly associated with *Citrus* species were used to test specificity of the real-time assay. A collection of the tested isolates, which are listed in Table 1, is kept at the authors' institutions on potato dextrose agar (PDA, Merck, Darmstadt, Germany) at 4°C and in 50% glycerol at −80°C.

Plant inoculation and fungal isolation

Six (first experiment) and 24 (second experiment) month-old, single-stemmed, potted seedlings of sour orange (*C. aurantium*) were inoculated on the stem-base by placing 50 µl of a conidial suspension (10^6 conidia ml^{-1} sterile water) on a wound made with a dissecting blade about 5 cm above soil level (Magnano di San Lio et al. 1992). Control seedlings were stem-inoculated with sterile water. The seedlings were grown in a screen-house under natural light at a temperature ranging from 15 to 25°C. At various time intervals after inoculation, seedlings were rated for symptom severity according to an empirical scale where: 0 = no symptoms; 1 = apical leaves chlorotic; 2 = epinasty or abscission of apical leaves; 3 = curling of both apical and expanded leaves and defoliation; 4 = wilting and collapse of the entire seedling.

The extent of xylem colonisation by the fungus was monitored by both molecular methods and isolation from the infected tissues on PDA to compare the sensitivity and the reliability of different techniques. At various time intervals (from 4 up to 28 days) after the inoculation, stem sections were cut at regular intervals of 1.5 cm (first experiment) and 20 cm (second experiment) from the inoculation point to the top. Each section was further split into two sub-samples that were used for molecular tests and for conventional isolation, respectively. To isolate the fungus, stem sections were surface-sterilised with Sial (Sial Chimica Catania, Italy), a commercial product containing 5–15% NaClO (corresponding to about 5% of active Cl), for 2.5 min. Stem pieces were then rinsed in sterile water, blotted dry on sterile filter paper and plated on PDA. The dishes were incubated at 22°C for up to 14 days in the dark. In each experiment, three replicate seedlings were sectioned at each time interval. Non-inoculated seedlings were used as a control. Each experiment was repeated twice.

Table 1 List of fungal species and isolates analysed in this study

Isolate	Species	Source	Location	Year
Pt VIII	*Phoma tracheiphila*	Unknown	–[1]	1982
Pt 42	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)[2]	1983
Pt 44	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 49	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 52	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 53	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 54	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 55	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 56	*P. tracheiphila*	*C. limon*	Bagheria (PA)	1983
Pt 60	*P. tracheiphila*	*C. limon* Femminello	C.da Baroni Noto (SR)	1988
Pt 61	*P. tracheiphila*	*C. limon*	C.da Bonavia Cassibile (SR)	1983
Pt 62	*P. tracheiphila*	*C. limon* Femminello	Balatelle Acireale (CT)	1983
Pt 63	*P. tracheiphila*	*C. limon* Femminello	Balatelle Acireale (CT)	1983
Pt 64	*P. tracheiphila*	*C. limon* Femminello	Balatelle Acireale (CT)	1983
Pt 71	*P. tracheiphila*	*C. limon* Monachello	Balatelle Acireale (CT)	1983
Pt 73	*P. tracheiphila*	*C. limon* Monachello	Balatelle Acireale (CT)	1983
Pt 75	*P. tracheiphila*	*C. limon* Monachello	Acireale (CT)	1985
Pt 77	*P. tracheiphila*	*C. limon* Monachello	Giardini (ME)	1985
Pt 79	*P. tracheiphila*	Air sampling	Ognina (CT)	1985
Pt 80	*P. tracheiphila*	Unknown	Giardini (ME)	1985
Pt 81	*P. tracheiphila*	Air sampling	Giardini (ME)	1985
Pt 83	*P. tracheiphila*	Air sampling	Giardini (ME)	1985
Pt 84	*P. tracheiphila*	*C. limon* Monachello	C.da Scorsonello Savoca (ME)	1988
Pt 86	*P. tracheiphila*	Air sampling	Ognina (CT)	1985
Pt 87	*P. tracheiphila*	Air sampling	Ognina (CT)	1985
ITEM 2338	*P. tracheiphila*	*C. limon* fruit	–	–
Pt C	*P. tracheiphila*	*C. microcarpa*	–	1983
Pt V	*P. tracheiphila*	*C. volkameriana*	–	1992
Pt 20	*P. tracheiphila*	Unknown	–	–
Pt Ad1	*P. tracheiphila*	*C. limon*	Altofonte (PA)	–
Pt Ad2	*P. tracheiphila*	*C. aurantium*	Altofonte (PA)	–
Pt Ad3	*P. tracheiphila*	*C. limon*	Parco d'Orleans (PA)	–
Pt Ad4a	*P. tracheiphila*	*C. aurantium*	Mazzarà Sant'Andrea (ME)	–
Pt Ad4b	*P. tracheiphila*	*C. aurantium*	Mazzarà Sant'Andrea (ME)	–
ISPaVe ER 1139	*P. tracheiphila*	*C. limon*	Cisterna (LT)	2000
PVS Pt S1	*P. tracheiphila*	*C. limon*	Capoterra (CA)	2004
ITEM 201	*P. glomerata*	*Laurus nobilis*	Italy	1981
ITEM 203	*P. exigua*	*Vitis vinifera*	Italy	1981
ITEM 243	*P. betae*	*Beta vulgaris*	The Netherlands	1966
ITEM 244	*P. cava*	*Castanea sativa*	The Netherlands	1966
ITEM 246	*P. fimeti*	Greenhouse soil	The Netherlands	1970
ITEM 2077	*P. lingam*	*Brassica napus*	Italy	1990
ISPaVe ER 693	*P. medicaginis*	*Medicago sativa* (seed)	Foggia (FG)	1991
PVS LB 3-2	*Diplodia aurantii*	*C. limon*	Sicily	2003
PVS A 3	*Phomopsis* sp.	*C. aurantium*	Bauladu (OR)	2003
PVS Fu A4	*Fusarium semitectum*	*C. aurantium*	Bauladu (OR)	2003
FS 2 B	*Fusarium solani*	*C. aurantium*	Gerbini (CT)	2001
FS R 2 B	*Fusarium solani*	*C. aurantium*	Gerbini (CT)	2001
LAT	*Fusarium solani*	*C. sinensis*	Gerbini (CT)	2001
Fox R 1 A	*Fusarium oxysporum*	*C. aurantium*	Gerbini (CT)	2001
Fox R 2 A	*Fusarium oxysporum*	*C. aurantium*	Gerbini (CT)	2001
FL	*Fusarium lateritium*	*Olea europaea*	Palermo, Sicily	1999

Table 1 (continued)

Isolate	Species	Source	Location	Year
C 2	*Colletotrichum gloeosporioides*	*Citrus* sp.	Calabria	1992
8 (JMO 94-22)	*Colletotrichum gloeosporioides*	*Citrus* sp.	California, USA	–
CP 3	*Colletotrichum gloeosporioides*	*C. limon*	Capo d'Orlando (ME)	1999
Acg	*Colletotrichum* sp.	*C. aurantium*	Bauladu (OR)	2003
PVS PD	*Penicillium digitatum*	*C. limon*	Sardinia	2003
PVS PI	*Penicillium italicum*	*C. limon*	Sardinia	2003
PVS LA 2-3	*Penicillium* sp.	*C. limon*	Sicily	2003
PVS E	*Epicoccum* sp.	*C. aurantium*	Bauladu (OR)	2003
GEO	*Geotrichum* sp.	*Citrus* sp.	Serravalle (CT)	2003
PVS B	*Botrytis cinerea*	*Malus domestica*	Sardinia	2003
PVS T 4-77	*Trichoderma harzianum*	Soil	Aglientu (SS)	2002
PVS-4	*Sclerotinia sclerotiorum*	*Foeniculum vulgare*	Sardinia	–
C 2	*Alternaria* sp.	*Tangelo 'Nova'*	Serravalle (CT)	2002
PVS MM	*Alternaria* sp.	*Citrus* sp.	Muravera (CA)	2002

[1] Not known.

[2] Code in brackets indicates the administrative province of Italian location: *PA* Palermo, *SR* Siracusa, *CT* Catania, *ME* Messina, *LT* Latina, *CA* Cagliari, *FG* Foggia, *OR* Oristano, *SS*, Sassari.

Purification of nucleic acids

DNA from inoculated and non-inoculated plants was obtained by grinding plant tissues in liquid nitrogen, taking an aliquot of 50 mg from the homogenate and then extracting the DNA by following a standard method (Aljanabi and Martinez 1997) slightly modified as follows: 200 µl of extraction buffer (50 mM Tris–HCl, pH 8.0; 2% SDS (sodium dodecyl sulphate), 0.75 M NaCl; 10 mM EDTA and 100 µg ml^{-1} proteinase K) were added to each sample and mixed well. Samples were incubated for 1 h at 65°C. Samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated by adding one volume of cold isopropanol. The pellet was then washed once with 100% ethanol and twice with 70% ethanol, re-suspended in 50 µl of TE (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) and stored at −20°C. All reagents were purchased from Sigma Aldrich (Milano, Italy).

A spin column-based extraction and purification protocol (Nucleo Spin Plant Macherey-Nagel GmbH, Düren, Germany) was adopted for DNA extraction from soil, by following manufacturer's instructions.

PCR primers and probe design

The primers and fluorogenic probe used in real-time PCR were designed using the Primer3 software (Rozen and Skaletsky 2000). Sequences of the ITS region were aligned by using GeneDoc v. 2.6.002 and compared with sequences available in the EMBL database for *P. tracheiphila* and other *Phoma* species as well as alignable sequences from anamorphic and teleomorphic *taxa* retrieved in BLAST searches (Altschul et al. 1997), using the consensus sequence of *P. tracheiphila* and other closest *taxa* available in databases as a query. The TaqMan® probe was labelled at 5′-terminal nucleotide with 6-carboxy-fluorescein (FAM) reporter dye and at 3′-terminal nucleotide with Black Hole Quencher (BHQ)-1. *In silico* PCR was performed for the primer-probe combination by using BLASTn against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) to ensure the specificity of the primers and probe prior to synthesis by Celbio s.r.l. (Pero, Milano, Italy).

Reference curve construction, quantification and data analysis

The reference curve for the extrapolation of results was constructed using standard values obtained by serial dilution of a plasmid harbouring the target insert. For this purpose, the 82-bp fragment of the ITS region was cloned by using the selected primers. This was achieved by amplifying the target sequence present in the extract using the conventional PCR conditions (95°C for 5 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 72°C for 10 min). The presence of the specific amplified

band was confirmed by electrophoresis in 2% agarose gel with ethidium bromide. The DNA was inserted in the PCR 4-TOPO vector of the TOPO TA Cloning Kit for Sequencing (Invitrogen, Leek, The Netherlands) using chemically competent *Escherichia coli* DH5-α colonies under the conditions recommended by the manufacturer. Purification of the plasmid DNA was performed using the Plasmid Midi Kit (Qiagen, Hilden, Germany), and the amount of plasmid DNA was determined by a spectrophotometer at 260 nm (GeneQuant proRNA/DNA Calculator, Amersham Biosciences, Piscataway, NJ, USA). Finally, 10-fold serial dilutions of the extract were prepared, ranging from 1 ng μl^{-1} (equivalent to 1×10^{8} plasmid copies μl^{-1}) to 1×10^{-8} ng μl^{-1} (equivalent to 1×10 plasmid copies μl^{-1}). Real-time PCR of the standard dilution series was performed in triplicate and yielded linear and reliable results ($R^2>$ 0.997).

The standard curve was generated by plotting the DNA amount (plasmid copies μl^{-1}) against the C_t value exported from the iCycler iQ Real-Time Detection System. The amount of DNA for unknown samples were extrapolated from the C_t value and the value obtained from the standard curve. Statistical analysis of real-time results were performed with the programme SPSS 12.0 for Windows (SPSS Inc. Chicago, Illinois).

Real-time PCR amplification

In the first experiment, the real-time PCR method with both the TaqMan® probe and the SYBR® Green I dye, was applied on the total DNA extracted from 6 month-old infected citrus at different stages of infection. In the second experiment, samples from 24 month-old infected citrus were tested only with SYBR® Green I dye. Real-time PCR was performed on the experimental samples and reference standards in triplicate and relative values for target abundance in each experimental sample were extrapolated from the standard curve generated from the reference standard. PCR was monitored on an iCycler iQ Real-Time Detection System. Reaction mixture of the real-time performed with the TaqMan® probe (25 μl total volume) contained 1 μl of template DNA dilution, 200 nM of Phomafor, 200 nM of Phomarev, 100 nM of Phomaprobe, 12.5 μl of 2X iQ Supermix (Biorad) and 10.5 μl of sterile bi-distilled water. Reaction mixture of the real-time performed with the intercalating dye SYBR® Green (25 μl total volume) contained 1 μl of template DNA dilution, 200 nM of Phomafor, 200 nM of Phomarev, 12.5 μl of 2X iQ SYBR® Green Supermix (Biorad) and 10.5 μl of sterile bi-distilled water. The PCR programme was as follows: 95°C for 3 min (denaturation, activation of polymerase and measuring of well factors), 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. After the real-time PCR performed with the SYBR® Green I, an additional melting curve was added to the reaction programme. A total of eight 10-fold dilution steps of plasmid standard (10 to 10^{8} target gene copies μl^{-1}) were run in triplicate on every plate, as well as a no-template control.

The primers, probe and PCR protocol were validated by amplifying DNA obtained from pure cultures of 36 *P. tracheiphila* isolates (Balmas et al. 2005). The specificity of the assay was tested on genomic DNA extracted from representative isolates of the following organisms: *Phoma glomerata, P. exigua, P. betae, P. cava, P. fimeti, P. lingam, P. medicaginis, Diplodia aurantii, Penicillium digitatum, P. italicum, Colletotrichum* sp., *Phomopsis* sp., *Fusarium semitectum, F. oxysporum, F. lateritium*, and *Citrus aurantium* (Balmas et al. 2005).

The sensitivity of the real-time assay with SYBR® Green I dye was validated by using total genomic DNA extracted from a titrated (10^{6} conidia in 500 μl sterile water) spore suspension of *P. tracheiphila* FC40, yielding a DNA concentration of 1.5 μg μl^{-1}. The extracted DNA sample was serially (10-fold) diluted in sterile water up to 1.5 pg μl^{-1}, and 1 μl of each DNA dilution was used as a template in a single reaction tube.

In order to estimate the interference of non-target fungal DNA in the real-time assay with SYBR® Green I dye, the same serial dilutions of *P. tracheiphila* FC40 DNA (i.e., ranging from 1.5 μg to 1.5 pg μl^{-1}) were mixed with a suspension containing 54 pg μl^{-1} of pooled genomic DNAs from the above-mentioned fungal species prior to amplification.

Finally, the effect of PCR inhibitors was tested by adding serial dilutions (from 1×10^{6} to 1 conidium in 500 μl sterile water) of a titrated spore suspension of *P. tracheiphila* FC40 to 50 mg of sterilised (121°C for 60 min on two successive days) potting mix (Humin-Substrat N17 Neuhaus, Klasmann-Deilmann, Geeste, Germany), finely ground with a mortar and pestle. The total DNA was either purified by following the standard protocol or by using the Nucleo Spin Plant kit (Macherey-Nagel GmbH) and 1 μl of each DNA sample was used as a template in a single reaction tube.

Table 2 Isolation of *Phoma tracheiphila* strain FC40 on potato dextrose agar from artificially inoculated 6 month-old sour orange seedlings (first experiment)

Distance (cm)	Days after inoculation							
	4		8		12		24	
	%	Arcsin√%±SE	%	Arcsin√%±SE	%	Arcsin√%±SE	%	Arcsin√%±SE
0–1.5	50.0	45±8.6	50.0	45±0	93.3	75.0±15.0	100	90±0
1.5–3.0	41.3	40±5.0	50.0	45±8.6	50.0	45.0±8.7	100	90±0
3.0–4.5	3.0	10±10.0	41.3	40±10.0	75.0	60.0±0	100	90±0
4.5–6.0	0	0±0	32.9	35±5.0	82.2	65.0±13.2	97.0	80±10.0
6.0–7.5	0	0±0	41.3	40±5.0	67.1	55.0±5.0	97.0	80±10.0
7.5–9.0	0	0±0	17.9	25±13.2	11.7	20.0±10.0	97.0	80±10.0
9.0–10.5	0	0±0	3.0	10±10	0	0±0	93.3	75±15.0
10.5–12.0	0	0±0	0	0±0	0	0±0	0	0±0

Results are expressed as the mean proportion (±SE) of stem fragments yielding the fungus at increasing distances from the point of inoculation sampled 4–24 days after inoculation. Each sample consisted of 12 stem fragments collected from three separate plants. Percentages were transformed into angular values for statistical analysis of data.

Results

Disease progress and isolation of the pathogen

The results of isolation from stem tissues of infected seedlings revealed a characteristic pattern of xylem colonisation by the pathogen. *P. tracheiphila* invaded the xylem progressively as shown by the decreasing frequency of positive isolations in non-symptomatic seedlings starting from the inoculation point to the apex. The fungus invaded the whole stem massively before foliar symptoms appeared. Both the rate of xylem colonisation by the fungus and the incubation period varied with the age of the seedlings.

In 6 month-old seedlings infected with *P. tracheiphila*, leaf symptoms appeared between 24 and 30 days after inoculation. The fungus was isolated 4 days after inoculation, but only from the basal portion of the stem (i.e., up to 4.5 cm from the point of inoculation). Similarly, 8 and 12 days after inoculation, a higher proportion of positive isolations was observed from the basal stem portions than from the apical ones. Twenty-four days after inoculation, almost all isolations were positive irrespective of the distance from the point of inoculation, thus indicating that the fungus had invaded the xylem (Table 2).

In 24 month-old seedlings, leaf symptoms did not appear until 31 days after inoculation. The pathogen was first isolated 21 days after inoculation and the frequency of isolation was inversely correlated with the distance from the point of inoculation. Similar results were obtained 28 days after inoculation (Table 3).

Table 3 Isolation of *Phoma tracheiphila* strain FC40 on potato dextrose agar from artificially inoculated 24 month-old sour orange seedlings (second experiment)

Distance (cm)	Days after inoculation							
	7		14		21		28	
	%	Arcsin√%±SE	%	Arcsin√%±SE	%	Arcsin√%±SE	%	Arcsin√%±SE
0–20	0	0±0	0	0±0	78.6	62.4±13.9	81.9	64.8±12.6
20–40	0	0±0	0	0±0	59.7	50.6±9.5	53.3	46.9±21.7
40–60	0	0±0	0	0±0	41.3	40.0±2.5	27.9	31.9±7.0

Results are expressed as the mean proportion (± SE) of stem fragments yielding the fungus at increasing distances from the point of inoculation sampled 7–28 days after artificial inoculation. Each sample consisted of 24 stem fragments collected from three separate plants. Percentages were transformed into angular values for statistical analysis of data.

Selection of primers and hybridisation probes

The alignment of the ITS region sequences of *P. tracheiphila* and of other *Phoma* species revealed several regions with low levels of homology between the different species. Six primers and two hybridisation probes, identified on the basis of the alignment, were chosen for optimisation of the PCR assay. The specificity of each primer-probe set was examined by performing PCR assays with a panel of DNAs from related and unrelated organisms (not shown). The primer pair that produced PCR products with the highest sensitivity and species specificity included Phomafor (5′-GCT GCG TCT GTC TCT TCT GA-3′) and Phomarev (5′-GTG TCC TAC AGG CAG GCA A-3′) that specifically amplified an 82 bp-long fragment of the ITS region quantified by the TaqMan® probe Phomaprobe (5′-F CCA CCA AGG AAA CAA AGG GTG CG Q-3′).

Sensitivity and specificity

The accuracy and precision of the real-time PCR assay were validated using serial dilutions of plasmid harbouring the target insert. The assay reliably detected 10 copies of the cloned target sequence. The fluorescent signal was proportional to the *log* concentration of the plasmid. Standard curves showed a linear correlation between input DNA and cycle threshold (C) values with an average PCR efficiency of 94.0%.

For the amplification of genomic DNA extracted from *P. tracheiphila* conidia, the standard curve was obtained by plotting Ct values versus the *log* DNA concentration of six 10-fold serial dilutions. The minimum amount of DNA that could be quantified was 15 pg, corresponding to a C_t value of 38.29 (Fig. 1a).

Furthermore, the assay did not cross-react with genomic DNA extracted from other fungi, but sensitivity was reduced, the standard curve being linear over five *log* units of initial quantities of template DNA, spanning from 1.5×10^{3} to 1.5×10^{-1} ng μl^{-1}, with a correlation coefficient (R^2) of 0.999 (Fig. 1b).

Total inhibition of the reaction occurred when conidia of the target pathogen were mixed with an organic soil substrate before extracting total DNA by using the standard protocol. The spin column-based alternative purification kit resulted in a significant decrease in sensitivity, the minimum amount of target

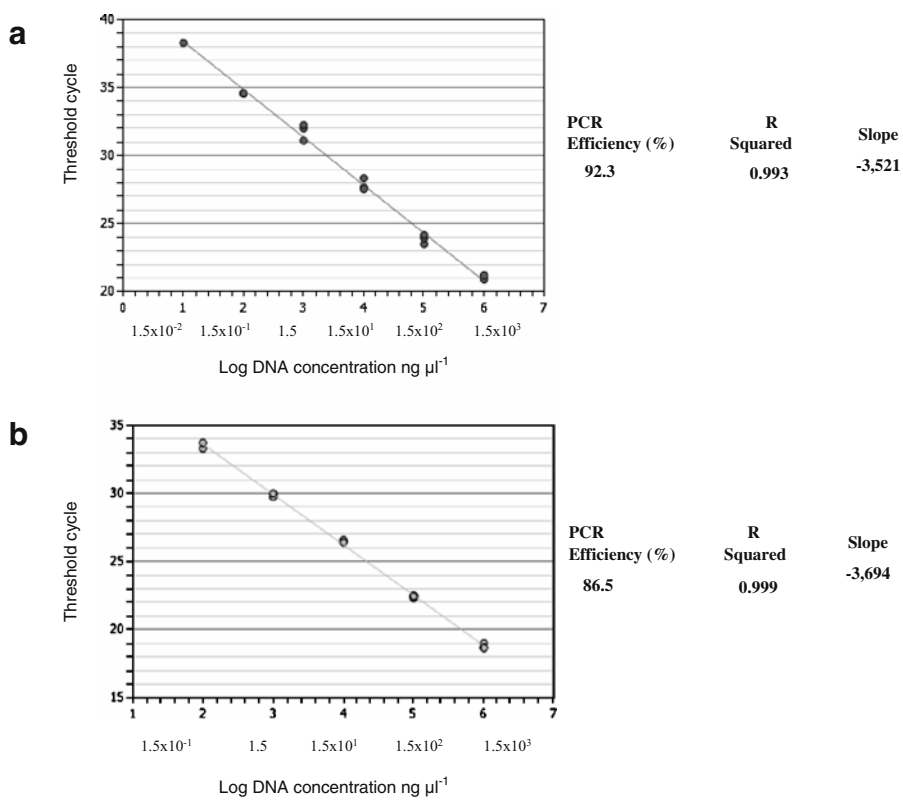

Fig. 1 Standard curves for the *in vitro* absolute quantification of *Phoma tracheiphila* using: **a**, genomic DNA extracted from conidia; **b**, genomic DNA of conidia mixed with a suspension containing 54 pg μl^{-1} of pooled genomic DNAs from non-target fungi. Standard curves were generated by plotting threshold cycle numbers (C_t value) *versus* the logarithmic genomic DNA concentration of each dilution series. The standard curve was linear over six *log* units of initial quantities of DNA spanning from 1.5×10^{3} to 1.5×10^{-2} ng μl^{-1} (**a**), and over five *log* units of initial quantities of DNA (1.5×10^{3} to 1.5×10^{-1} ng μl^{-1}) (**b**), respectively

DNA to be accurately quantified corresponding to 950 pg.

Detection of *Phoma tracheiphila* in artificially inoculated sour orange seedlings

The target sequence was not detected by real-time PCR in DNA samples extracted from uninfected *Citrus*, while detection was achieved in samples tested after inoculation with *P. tracheiphila*, including apical stem pieces from which both conventional isolation on PDA and standard PCR were negative.

In the first experiment, all the samples from 6 month-old seedlings were tested with SYBR® Green I and TaqMan® technology in order to compare the two methods. Target sequence concentration values were similar at the 4th and the 8th day following inoculation and were consistent with the results of conventional isolation and standard PCR. Values were inversely correlated with the distance from the inoculation point. A distinct increase in target copies was observed with both chemistries at the 24th day, corresponding to the invasion of the xylem by the fungus (Fig. 2). There was no significant difference between the TaqMan® and SYBR® Green I methods (R^2=0.98; P<0.0001). This result indicates that the SYBR® Green-based assay is as sensitive as the TaqMan® assay with the same PCR primers.

Since SYBR® Green I indiscriminately binds to double-stranded DNA, other products in the PCR such as primer dimers may be detected along with the target gene. To verify that the SYBR® Green I dye detected only one PCR product, the samples were subjected to the heat dissociation protocol following the final cycle of the PCR. Dissociation of the PCR reactions consistently produced a single peak, demonstrating the presence of only one product in the reaction (Fig. 3).

The assay with the intercalating dye is less expensive and simpler than TaqMan® assay in its manipulation and should be more appropriate for a large routine. For

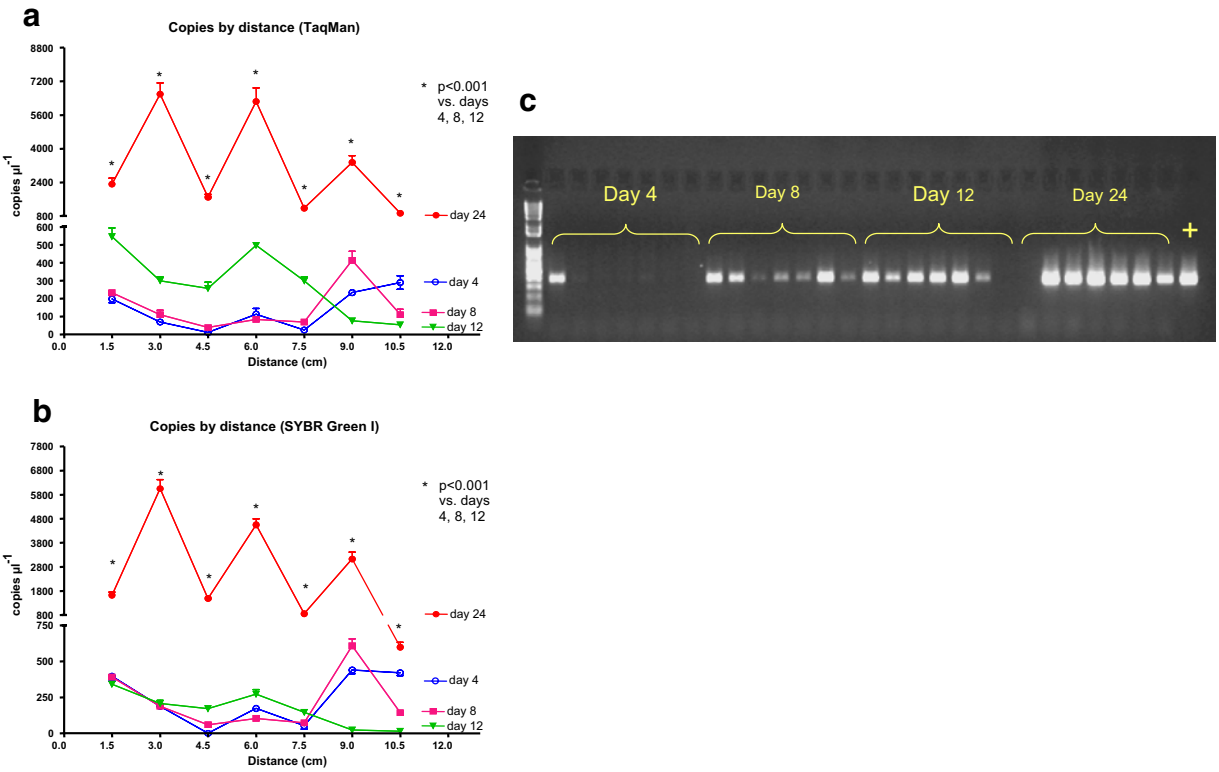

Fig. 2 Comparative detection of *Phoma tracheiphila* in artificially inoculated 6-month-old *Citrus aurantium* seedlings (First experiment) by real-time PCR with TaqMan® (**a**) or SYBR® Green I (**b**) chemistries and by standard PCR (**c**). The concentration of target sequence in all the infected samples was plotted against the distance, expressed in cm, from the point of inoculation. Standard PCR was performed using primers PtFOR2 and PtREV2, and positive reactions showed a single amplification product of 378 bp

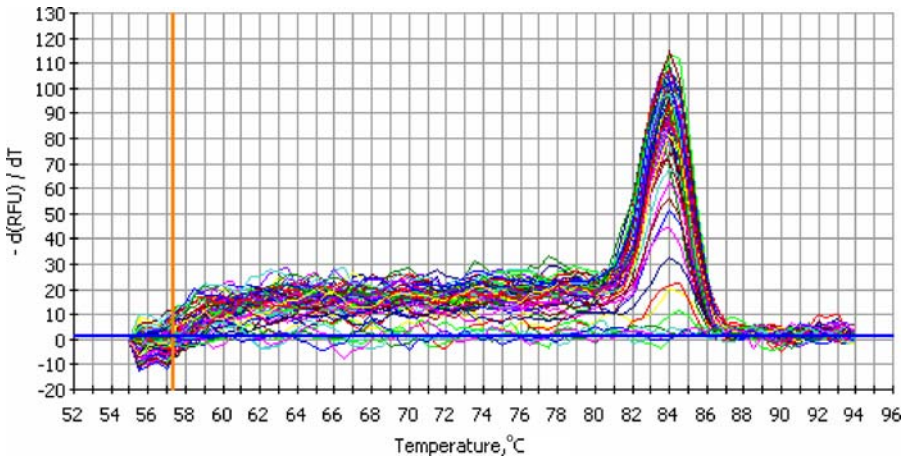

Fig. 3 Melting curve (fluorescence *versus* temperature) of amplification products obtained from the first experiment (6 month-old *Citrus aurantium* seedlings artificially inoculated with *Phoma tracheiphila*). The melting temperature of the target amplicon occurs at 84°C. No contaminating products are present in the reaction

this reason, in the second experiment, samples from 24 month-old infected citrus were tested only with SYBR® Green I dye.

In 24 month-old sour orange seedlings infected by the FC40 isolate of *P. tracheiphila*, the pathogen was detected from all stem sections collected at 14, 21 and 28 days after inoculation, while both the conventional isolation method and the standard PCR were able to detect the pathogen only after 21 days. The concentration of the pathogen increased with time and the maximum value was observed 28 days after inoculation in the apical section but decreased with distance from the inoculation point 14 and 21 days after inoculation (Fig. 4). This pattern of fungal DNA concentration in the xylem of the stem is consistent with that determined with the conventional isolation method. Statistical analysis of the results obtained in the second experiment showed that the effect of time (days) was highly significant ($P<0.0001$), accounting for 44.8% of total variance, while distance ($P<0.0001$) accounted for 17.8% of total variance.

Discussion

The 'mal secco' disease of citrus may have a long incubation period which varies depending on several including plant age and susceptibility, virulence of the pathogen isolate as well as environmental factors (Solel 1976; Perrotta and Graniti 1988; Magnano di San Lio et al. 1992; Cacciola et al. 1996). Furthermore, *P. tracheiphila* may cause latent, chronic infections, which can induce the symptoms of the disease when triggered by changes in environmental conditions or physiology of the host plant. For these reasons, *P. tracheiphila* is internationally recognised as a dangerous plant pathogen and, quite interestingly, it has been included in the list of 'Animal and plant pathogens with potential biological warfare applications' (Lillie et al. 2005).This study aimed at developing tools that may be used to diagnose latent infections precisely and to follow the time-course of the invasion of plant tissues by *P. tracheiphila*.

Conventional tests used to detect *P. tracheiphila in planta* involve isolation on nutrient substrates and morphological characterisation, but the disadvantage of such methods is that accurate pathogen identification can be difficult and time-consuming. More recently, PCR-based techniques were developed for the diagnosis of *P. tracheiphila* (Balmas et al. 2005). Despite its versatility, standard PCR may not be sensitive enough to guarantee plant material as being free from the pathogen; moreover, this approach is not quantitative. Recently, methods based on real-time PCR have become widespread in the clinical field for the detection of microbial infections (Bruijnesteijn Van Coppenraet et al 2004; Hussain et al. 2006). Reports of real-time PCR application in plant pathology are constantly increasing, because of its sensitivity and specificity, and for the possibility to quantitatively detect plant pathogens (Lopez et al. 2003; Schaad and Frederick 2002; Ward et al. 2004). This technique eliminates the requirement for post-amplification processing steps and significantly reduces time and labour. Furthermore, avoiding the need for ethidium bromide manipulation, health risks for operators and environmental contamination are reduced.

In this study, a real-time assay based on TaqMan® chemistry was developed in order to achieve specific

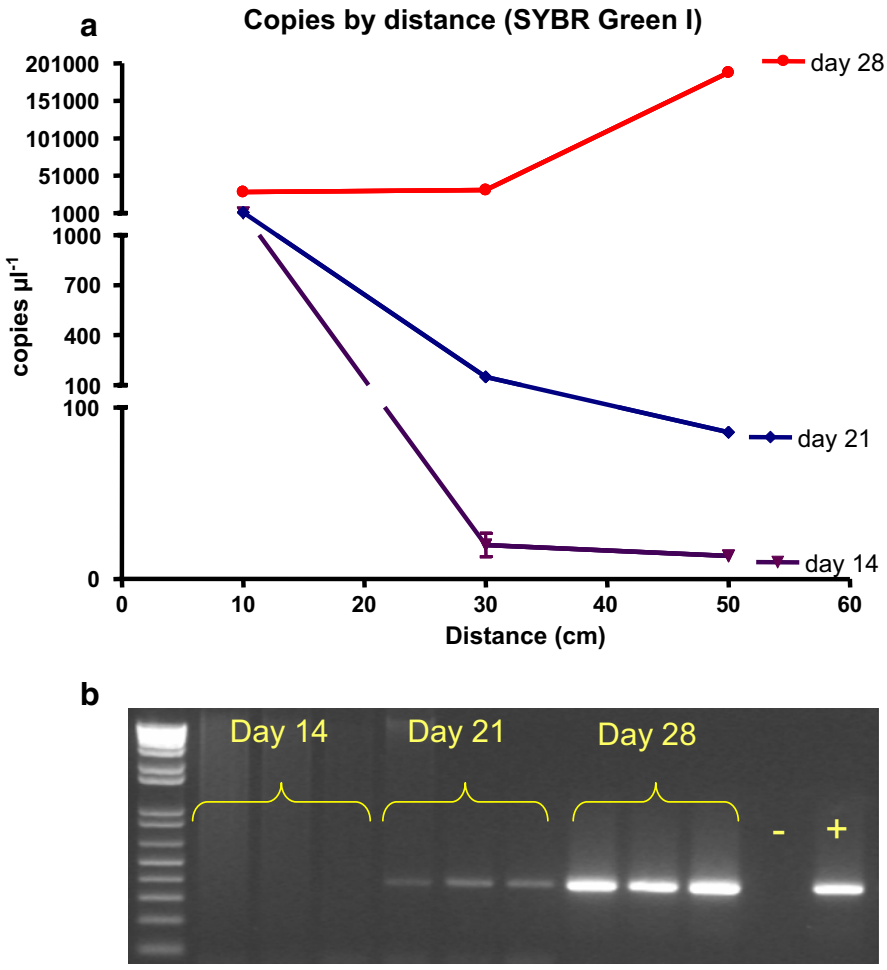

Fig. 4 Comparative detection of *Phoma tracheiphila* in artificially inoculated 24 month-old *Citrus aurantium* seedlings (Second experiment) by real-time PCR using SYBR® Green I chemistry (**a**) and by standard PCR (**b**). The concentration of target sequence in all the infected samples was plotted against the distance, expressed in cm, from the inoculation point. Standard PCR was performed using primers PtFOR2 and PtREV2, and positive reactions showed a single amplification product of 378 bp

and sensitive detection and quantification of *P. tracheiphila* in plant material: the TaqMan® assay was compared with the SYBR® Green I assay, with the conventional PCR assay, and with the classic microbiological approach to detect the plant pathogen, in order to evaluate the most reliable method for 'mal secco' diagnosis.

Because rRNA genes in fungi are often found as tandem repeats of up to 100–200 copies (Maicas et al. 2000; O'Sullivan et al. 2003), a reaction with 1,000 rDNA copies was considered to correspond to 10 genome equivalents. Early observations on the nuclear condition of *P. tracheiphila* suggest that most pycnospores and phyaloconidia are mononucleate, although conidia bearing two or three nuclei were observed at low (usually 5%) frequency (Magnano di San Lio and Graniti 1987). The real-time assay developed in this work reliably detected 10 cloned copies of the target rDNA sequence, corresponding to hypothetical sensitivity of 1/10–1/20 of haploid genome (or mononucleate spore). When the real-time SYBR® Green I assay was tested with serially diluted DNA extracted from a titrated spore suspension of the target pathogen, the minimum amount detectable was 15 pg, corresponding to <1 fungal spore per reaction.

Licciardello et al. (2006) reported on the development of a *P. tracheiphila*-specific quantitative real-time assay by using the TaqMan® chemistry. The minimum amount of pathogen DNA that could be quantified accurately in the assay was 1 pg (Licciardello et al. 2006). Sharing a similar level of sensitivity, the assay described here and the real-time method developed by Licciardello and co-workers should be carefully validated in ring tests to compare their sensitivity and specificity with a common set of biological samples.

Our results indicate that both the TaqMan® and SYBR® Green I assays proved sensitive and specific, and the results obtained with the two chemistries were

consistent and equivalent, although the TaqMan® technology was more expensive and difficult to set up. A common advantage of both molecular methods based on real-time PCR is their rapidity. The time needed to obtain definitive results with molecular methods is less than one working day. Conversely, isolation on agar media requires at least 7 days, but a 2-week incubation is recommended to obtain definitive results, as the number of positive isolations may increase (data not shown). Owing to their sensitivity, molecular methods appear more suitable than conventional isolation methods for detecting *P. tracheiphila* infections in *Citrus* plants, which are colonised by the pathogen discontinuously, leading to less consistent isolation. This could prove particularly useful for detecting *P. tracheiphila* in young plants with latent infections or in chronically infected adult plants, such as those affected by the *facies* of disease known as 'mal nero.'

As far as the epidemiology of 'mal secco' disease is concerned, the inconvenience of false negatives is particularly relevant if the assay is aimed at monitoring the inoculum of *P. tracheiphila* in plant debris in the soil, as a variety of naturally-occurring compounds, such as humic acids, tannins and lignin-associated compounds, can interfere with PCR reactions and inhibit the amplification (Bridge and Spooner 2001; Cullen and Hirsch 1998). Aiming at evaluating the efficacy of the real-time method under different conditions, we have experienced the complete inhibition of the reaction when conidia of the target pathogen were mixed with an organic substrate before extracting total DNA by using the standard protocol. Thus, an alternative extraction and purification protocol through spin columns was needed, resulting in a drastic decrease in sensitivity. Therefore, a method for the prior assessment of DNA quality is essential despite recent improvements in DNA extraction methods (Bridge and Spooner, 2001; Cullen and Hirsch, 1998). This aspect is particularly important for quarantine pathogens such as *P. tracheiphila*, for which results of a molecular analysis could impact upon large-scale eradication schemes or trade.

The relative simplicity and high sensitivity of real-time suggest it might be of great benefit for rapid diagnosis of 'mal secco' disease in epidemiological studies, pathogen surveys, breeding and selection programmes for disease resistance, as well as for quarantine purposes.

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